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The Murine Toxin of *Pasteurella pestis*: A Study in Its Development

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INTRODUCTION

Plague, for which the organism *Pasteurella pestis* is responsible, has been a serious problem for mankind through each epoch of his existence. Today, despite great knowledge and technology, many world areas still suffer seriously from this disease. In the late 1940's and early 1950's, a series of investigations on plague toxin was initiated by Ajl and co-workers, and continued to the present. These studies, which provided a renewed approach to the pathogenesis of plague, form the basis of this review.

Certain aspects of the symptomatology and pathology of the disease prompted Dieudonne and Otto (16) in 1928 to suggest that toxic substances of considerable potency are liberated by *P. pestis* during infection. Additional interest in the role of toxin in the disease was generated by the findings of McCrumb et al. (53), in Madagascar, that antibiotics administered 36 to 48 hr after the onset of the disease failed to save patients despite the fact that, on autopsy, the blood and organs were sterile.

The plague bacillus is known to contain several distinct and readily separable antigenic components. Our interests, however, have remained in the toxin produced by *P. pestis*, which is specifically responsible for death of mice and rats and is known, therefore, as the "murine" toxin.

It is not the purpose of this review to cover completely the available literature on plague toxin. We shall trace here the development of the work in our laboratory with the murine toxin of *P. pestis* and refer to the investigations of others only as they relate to and amplify our studies.

The primary objectives from the start of our work were isolation and purification of the toxin and study of the mechanism of its action at an enzymatic level. Recently, we have concerned ourselves with how this toxin is synthesized by the bacterium, the regulation of this synthesis, and the distribution of this protein in the *P. pestis* cell.

The murine toxin of *P. pestis* has been obtained in a highly purified form. However, we found, as have others working with certain exotoxins (for example, diphtheria toxin), that the toxic activity of highly purified preparations is associated with more than one component. Our purest preparations are composed of two distinct molecular species, each exhibiting essentially identical toxic activity.

Studies on the mode of action of the toxin involve primarily the effects of the toxin on mammalian mitochondria. An association between the ability of the toxin to inhibit mitochondrial respiration and its action in vivo has been established. The site of inhibition of mitochondrial respiration resides in the electron transfer chain in the region between reduced nicotinamide adenine dinucleotide ($NADH_2$) or succinate and cytochrome *b* and, more specifically, at the $NADH_2$-coenzyme Q reductase complex. A permeability

phenomenon is also involved in the inhibitory effect of the toxin on mitochondrial respiration. This is correlated with the toxin's ability to induce mitochondrial swelling and to interfere with respiration-dependent mitochondrial ion accumulation.

Concerning the mode of synthesis of this toxin by *P. pestis*, it was found that tryptophan analogues selectively inhibit toxin biosynthesis, and that this inhibitory effect can be reversed by intermediates of tryptophan biosynthesis in microorganisms. This finding suggests a tryptophan requirement for toxin formation.

ISOLATION AND PURIFICATION OF THE TOXIN

Studies on the biological action and antigenic behavior of plague toxin were handicapped by the lack of adequately pure material. It was known that the toxin of *P. pestis* is associated with the cells and must be liberated from them. Numerous procedures, including cell lysis, sonic vibration, and chemical extraction, were employed to liberate the toxin from cells. Markl (51, 52) used filtrates of old broth cultures. Girard (21) employed a freezing and thawing system, and Jawetz and Meyer (31) held agar-grown suspensions at 37 C for 48 hr and then at 4 C for 24 hr.

Sonic vibration was used successfully by Smith et al. (68) to obtain highly toxic material from *P. pestis*. Extraction of dried cell powders or whole cells with relatively simple compounds was employed by Baker et al. (7, 8) with sodium chloride, by Lustig and Galeotti (48) with 1% potassium hydroxide, and by Goodner et al. (22) with sodium deoxycholate.

These toxin preparations obtained were crude, for they contained numerous antigenic components in addition to the toxin. Initial attempts to purify these toxic materials consisted of precipitating them with either ammonium sulfate (8) or calcium chloride (79) and freeing them from the nonprecipitable fractions. Baker et al. (8) treated toxin, prepared by extracting acetone-dried cells of *P. pestis* with 2.5% sodium chloride, with 30 and 40% saturated ammonium sulfate to remove antigens other than the toxin as the residues. The toxin was also precipitated directly by the addition of 55 to 67% saturated ammonium sulfate. The best samples, having an LD_{50} for 20-g mice of 0.6 to 0.8 μg, represented a 10-fold concentration of the toxin. However, all the toxic preparations were heavily contaminated with atoxic soluble antigens, and attempts at further purification resulted in a 50 to 75% loss in toxicity.

Englesberg and Levy (18) obtained highly toxic fractions by precipitating the crude toxin obtained from autolysates of *P. pestis* grown at 30 C in semisynthetic medium (casein hydrolysate-mineral-glucose medium) with saturated ammonium sulfate, followed by dialysis and lyophilization. Though the relative purity of this material was not given, this method, compared with previous attempts, provided substantially greater yields of toxin.

Ajl et al. (1) undertook the first extensive study designed to obtain preparations of plague toxin which were pure by all of the known standards employed to ascertain protein purity. The initial phase of this work considered purification of the toxin by chemical means, involving extraction of the toxin from acetone-dried cells of the avirulent "Tjiwidej" (TJW) strain of *P. pestis* with 2.5% sodium chloride, followed by ammonium sulfate and isoelectric precipitations for partial separation of the toxin from the envelope substance. This preparation was then treated with manganese chloride for removal of nucleic acids, with methanol precipitation to concentrate protein and remove extraneous materials and calcium phosphate gel absorption with elution to separate further the toxin from the envelope substance. Lipoid materials were removed by chloroform extraction. The final material, having an intraperitoneal LD_{50} of 2.6 μg for 16- to 18-g mice, exhibited a sevenfold increase in toxicity. However, it contained one major and frequently one or more minor components when observed in the analytical ultracentrifuge.

The high resolving power for fractionating protein mixtures afforded by continuous-flow paper electrophoresis led to its utilization (1) for further purification of the toxin. Material obtained from the final stage of the chemical purification procedure was passed twice through a paper electrophoresis cell described by Durrum (17). Electrophoretic patterns showed that this material was considerably more pure than that obtained by chemical procedures alone. Purified toxin with an isoelectric point of 4.7 behaved as a homogenous protein in the ultracentrifuge and Tiselius electrophoresis cells and was free from carbohydrates, nucleic acids, and capsular antigen. Sedimentation and diffusion data indicated a molecular weight for the toxin in the order of 74,000.

This toxin was subjected (3) to the very sensitive gel diffusion precipitation reactions of Oudin (60) and Ouchterlony (61), and at least two and frequently three or more individual zones of precipitation were found. Since the main use for this toxin was to be the determination of the mechanism of its action at an enzymatic level, it was imperative to achieve an even greater degree of purity. As considerable trauma to the toxin mole-

cule was involved in chemical extraction procedures, the method was simplified considerably. Crude toxin was fractionated with ammonium sulfate, and the dialyzed fraction between 35 and 70% saturated ammonium sulfate was passed several times through the continuous-flow, hanging-curtain electrophoresis apparatus developed by Karler (39). The first two passes were in Veronal buffer, 0.01 ionic strength (*p*H 8.6), with subsequent passes in maleate buffer, 0.01 ionic strength (*p*H 6.0). After final passage, a 17-fold purification of the toxin was achieved. This final material exhibited only one band against crude rabbit antisera in the Oudin reaction and had an intraperitoneal LD_{50} for 14- to 18-g Swiss albino mice of 0.7 μg and an intravenous LD_{50} of less than 0.2 μg. Similar results were obtained with toxin from virulent strains of *P. pestis* by Spivack and Karler (69). Their material had an intravenous LD_{50} for mice of 0.1 μg.

Extremely pure preparations of toxin were obtained with the Karler electrophoresis apparatus. This procedure, however, is characterized by slow rates of separation of protein components, entailing hours or days needed for fractionation of large volumes of dilute protein solutions. There also may be losses of sample due to adsorption on paper, the supporting medium.

A transparent methyl methacrylate cell packed with fine glass beads was developed by King, Jensen, and Stubbings (32, 40) for preparative electrophoresis, and was modified and refined for general electrophoretic separatory procedures. Since large quantities of toxin are required for studies involving the mechanism of toxin action, the continuous-flow electrophoresis apparatus employing glass microbeads, fractionating large volumes of material in short periods with insignificant adsorption of sample upon the glass bead matrix, was chosen for purifying plague murine toxin. Crude toxin, obtained from autolyzed *P. pestis* cells and fractionated with ammonium sulfate between 35 and 70% saturation, was passed through the electrophoresis apparatus according to the procedure reported by Winsten et al. (77) using serum proteins. The toxin so obtained had an intraperitoneal LD_{50} for 16- to 18-g Swiss albino mice of approximately 2 μg of protein, and it contained 95% protein.

To determine whether plague toxin possessed any unusual components which could account for its high toxicity, detailed elemental and amino acid analyses were performed (9) on the most highly purified samples of toxin available. Eighteen amino acids and a number of elements were identified. On a dry-weight basis, over 98% of the toxin molecule was accounted for by organic analysis, including ammonia and ash content. There was nothing unusual about the toxin molecule aside from the high proportion of acidic amino acids, which verified the previously observed isoelectric point of the toxin of 4.7.

IMMUNOLOGICAL PROPERTIES

Before purified *P. pestis* toxin was available, its role in immunity against plague was largely conjectural. The purified preparations of Ajl et al. (1) provided an excellent source of antigen to be used for detailed investigations of the immunological properties of plague toxin. Warren et al. (76) utilized the most purified preparations of Ajl et al. (1) obtained from the TJW strain of *P. pestis* to produce antitoxin in rabbits. The antiserum obtained was able to flocculate, hemagglutinate, and fix complement with toxin and toxoid.

Additional investigations revealed that the specific TJW antitoxin neutralizes toxins obtained from different strains of *P. pestis*. This finding and the observation that purified TJW toxin reacts with the antisera prepared from a variety of avirulent and virulent strains of the plague bacillus suggest strongly that all these toxins have similar antigenic structures.

To determine the degree of animal immunization against toxin (2), mice were challenged intraperitoneally with formalin-treated toxin and intravenously with toxin-antitoxin mixtures. In the former case, mice were protected against 60 to 80 LD_{50} doses of the toxin, and in the latter only a few LD_{50} doses of the toxin were neutralized.

The role of toxin in plague infection has been studied by Meyer (*unpublished data*), who found that rats and guinea pigs died in shock when injected with soluble toxins or with killed and dried or living *P. pestis* cells. The liver and spleen served as filters which removed *P. pestis* organisms from the blood after intravenous injection. Bacilli were destroyed in the liver and released toxic materials responsible for the intoxication. The total amount of bacillary somatic antigen was related directly to the speed with which the symptoms of intoxication were noted.

It has been tacitly assumed that a definable toxic material is present in the circulating blood in the course of plague infection. However, its role in the pathophysiology of the disease has not been elucidated. In a fatal case of plague, many different events, all related, proceed so rapidly that it is difficult to ascertain the dominant factor. As it is difficult to determine the order of these physiological events and to identify factors responsible for these events, the role of toxin in

plague infection is certainly not completely understood.

MECHANISM OF ACTION OF THE TOXIN

Effect of the Toxin on Microbial Extracts and Crude Tissue Homogenates

The first serious attempt to study the mechanism of action of toxin on an enzymatic level involved the effect of toxin on oxidation of a variety of substrates by cell-free microbial extracts and crude mouse liver homogenates. It was found (5) that the toxin specifically inhibited oxidation of α-keto acids. For example, whereas the toxin inhibited oxidation of α-ketoglutaric and pyruvic acids, it exhibited a significantly lesser effect on such acids as succinic and citric.

Similar results were obtained with heat-inactivated and formalin-treated toxin. These findings can be understood only when compared with results of investigations concerning the effect of inactivated toxin on mitochondrial respiration. This discussion follows in a subsequent section.

The inhibitory effect of toxin on the oxidation of α-keto acids by cell-free microbial extracts and crude mouse liver homogenates was reversed by the addition of an excess of nicotinamide adenine dinucleotide (NAD) but not by nicotinamide adenine dinucleotide phosphate (NADP). This finding suggests that the toxin possesses nicotinamide adenine dinucleotidase activity and thereby interferes with certain NAD-dependent reactions by depriving them of the required coenzyme. Thus, when this toxin was incubated with NAD in the presence of phosphate and the reaction products were analyzed (4), three compounds were recovered, namely, nicotinamide mononucleotide (NMN), adenylic acid, and a compound similar to, but not identical with, the classical adenosine diphosphate (ADP). In the absence of phosphate very little NAD was broken down. This was to be expected, since the formation of NMN and a compound similar to ADP per mole of NAD cleaved requires the incorporation of an additional phosphate group from the reaction medium. The requirement for phosphate classified this reaction as an "unusual" type of nicotinamide adenine dinucleotidase reaction, for neither of the following classical ways by which NAD is cleaved enzymatically is phosphate-dependent: at the pyrophosphate linkage to yield adenylic acid and NMN (41) or at the nicotinamide ribose linkage to form nicotinamide (24) and adenosine diphosphoribose (38).

Toxin preparations used to study nicotinamide adenine dinucleotidase activity and inhibitory effects on α-keto acid oxidation were purified by chemical and electrophoretic procedures of Ajl et al. (1). When serologically homogenous toxin was obtained (3), each of these properties was reinvestigated and it was found that the activity on NAD disappeared, whereas the characteristic α-keto acid inhibition remained. The nicotinamide adenine dinucleotidase activity was recovered in a different protein fraction. The electrophoretic mobility of this protein was very similar to that of the toxin.

Although nicotinamide adenine dinucleotidase (NADase) activity does not appear to be associated with mode of action of plague murine toxin, an interesting NADase has been uncovered. Further investigations relative to the mechanism of action of this enzyme are warranted.

Action of the Toxin on Mammalian Mitochondria

Effect on respiration. It was important to determine toxin action at a higher level of organization from the standpoint of enzymatic structure and closer to that expected to occur in the animal while still maintaining an in vitro system. Toward this goal investigations were undertaken to study toxin effect on mitochondria, which are known to be the active sites of respiration in animal tissues. A critical observation, providing an insight to understand the susceptibility and resistance of animal species to biological poisons, was in the association present between the ability of toxin to inhibit mitochondrial respiration from certain species and the susceptibility of these animals to in vivo action of the toxin.

TABLE 1. *Effect of plague toxin, Vi and O antigens, and bovine serum albumin on the respiration of heart mitochondria**

Source of mitochondria	Additions	O_2 consumed†	Per cent inhibition
Rat heart	None	0.72	—
	Boiled plague toxin (2.5 mg)	0.66	8.3
	Bovine serum albumin (2.5 mg)	0.72	0.0
	O antigen (2.5 mg)	0.65	9.7
	Plague toxin (1.0 mg)	0.24	66.7
Rabbit heart	None	0.41	—
	Vi antigen (2.5 mg)	0.41	0.0
	O antigen (2.5 mg)	0.50	0.0
	Plague toxin (2.5 mg)	0.44	0.0

* This table was compiled from results given in reference 62.

† Expressed as micromoles per liter per second.

It is known that the toxin is lethal for the mouse and rat but not for the rabbit, chimpanzee, dog, or monkey. Therefore, we were interested to learn that toxin inhibited the respiration of heart mitochondria from the toxin-susceptible rat and mouse, but had no effect on similar preparations from the toxin-resistant rabbit (Table 1). Additional experiments revealed that the toxin was unable to inhibit respiration of heart mitochondria from chimpanzee, dog, and monkey (66). Only the exogenous mitochondrial respiration of toxin-susceptible animals was inhibited. The endogenous respiration remained unaffected. Likewise, oxidative phosphorylation was in no way altered by the toxin. The inhibitory effect on mitochondrial respiration was specific for the toxin investigated, since bovine serum albumin, representing another protein, and the Vi and O lipopolysaccharide antigens had no effect on mitochondrial respiration.

Toxin action varied not only with respect to the species yielding the mitochondria but also with respect to the specific organ from which they were isolated. It was found that toxin had no effect on the respiration of brain mitochondria of toxin-susceptible rats. Studies on the effect of toxin on liver mitochondria revealed that the toxin inhibited the respiration of liver mitochondria from the rat and rabbit to the same extent as rat heart mitochondria (35). This inhibition of rabbit liver mitochondrial respiration is of interest, since other studies indicate that 10 mg of toxin, injected intraperitoneally, is not lethal for 2-kg rabbits.

Although physiological concentrations of toxin were found to inhibit mitochondrial respiration, toxin treated in any manner that made it atoxic for the animal resulted in the concomitant loss of ability to inhibit mitochondrial respiration (see Table 1). This contrasted directly with results obtained with cell-free bacterial extracts and crude tissue homogenates. This inconsistent behavior of inactivated toxin can be explained as follows. One basic characteristic of this toxin, which is its ability to inhibit the oxidation of certain compounds, remains with the molecule even when toxicity is lost. When homogenates are employed, the inhibition of keto acid oxidation reflects chance interaction of the toxin or toxoid with dispersed enzyme molecules. The active portion of the toxin molecule for this effect remains after heating or formalin treatment of the molecule. However, the mitochondrion retains a relatively high degree of organization. In this case the effects depend upon critical spatial relations, and, to exert these effects, the toxin molecule must retain not only that portion reacting with the enzyme, but also the portion permitting effective orientation within the structure of the mitochondrion.

The inability of inactivated toxin to inhibit mitochondrial respiration demonstrated a correlation between toxicity of the toxin in vivo and its in vitro action on mitochondria and led to the hypothesis that inhibition of mitochondrial respiration may explain the action of this toxin in vivo.

As this toxin inhibited heart mitochondrial respiration of toxin-susceptible species, the hypothesis above would indicate heart malfunction as an early sign of toxin action in susceptible species. Heart malfunction is detected easily by electrocardiographic measurements. Indeed, alterations did occur in the S-T segment of the electrocardiogram of the rat within 60 min after injection of lethal or sublethal doses of toxin and prior to any changes in hematocrits or blood pressures. In animals surviving sublethal doses of the toxin, electrocardiographic changes observed initially were no longer evident after 24 to 48 hr or after the animal had recovered completely. Similar electrocardiographic changes did not occur in rats dying from hemorrhagic shock, hypoxia, glucose intoxication, or in toxic deaths from *Escherichia coli* endotoxin. Toxin-resistant rabbits exhibited no alterations in their electrocardiographic tracings when injected with toxin doses up to 50 mg. Thus, whenever the toxin inhibited heart mitochondrial respiration, corresponding changes in electrocardiographic patterns obtained from intoxicated animals were found. Conversely, failure to observe such in vivo effects was associated with unaltered mitochondrial respiration.

If the inability of the toxin to inhibit the respiration of heart mitochondria derived from the toxin-resistant rabbit were due to the exclusion of toxin by the rabbit heart mitochondrial membrane, disruption of these mitochondria should result in inhibition of their respiration with addition of toxin comparable to that obtained with intact heart mitochondria from the toxin-susceptible rat. Experimentally, mitochondria were disrupted chemically with deoxycholate and physically by means of sonic vibration, and it was found that, although toxin had little or no effect on respiration of unaltered rabbit heart mitochondria, respiration of disrupted mitochondria was inhibited to a significant extent (35).

Toxin-susceptible animals can be immunized against toxin. When mitochondria were isolated from the hearts and livers of such immunized animals and incubated with the toxin, it was demonstrated that the inhibitory effect of toxin on heart mitochondrial respiration was reversed, whereas the respiration of liver mitochondria of

TABLE 2. *Effect of toxin on swelling of heart, liver, and brain mitochondria**

Source of mitochondria	Toxin concn	Net change in optical density at 520 mμ of experimental minus control†
	mg	
Rat heart	2.0	0.325
	0.5	0.210
Rabbit heart	2.0	0.025
	0.5	0.020
Rat liver	2.0	0.243
	0.5	0.120
Rabbit liver	2.0	0.235
	0.5	0.170
Rat brain	2.0	0.020

* This table was compiled from results given in references 34 and 35.

† Change in optical density at 520 mμ recorded after 30 min of incubation.

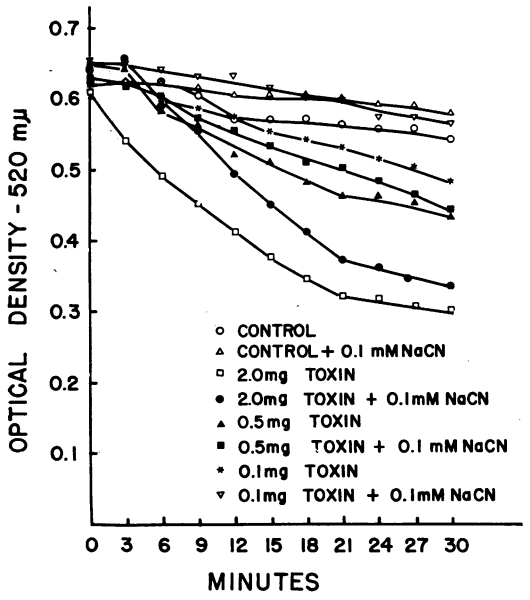

FIG. 1. *Effect of NaCN on toxin-induced swelling of rat heart mitochondria. From Kadis and Ajl (34).*

immunized and nonimmunized animals was inhibited to the same extent (35).

Ability of the toxin to induce mitochondrial swelling. Mitochondria, primary sites of oxidative metabolism, also possess the following characteristic properties: ability to cause transport and accumulation of certain electrolytes and ability to take up water and swell and to extrude water and contract. These transport processes are dependent upon respiratory energy and are associated with electron carriers and coupling enzymes of the respiratory chains, present in and constituting a large portion of the protein layer of the mitochondrial membrane.

A wide variety of agents are known to cause mitochondrial swelling (44). One type, known as electron transport-dependent swelling, is thought (30) to depend upon increased membrane permeability which stops short of osmotic rupture of the mitochondria. The toxin under consideration does not inhibit the respiration of intact rabbit heart mitochondria as the membranes of these mitochondria apparently exclude the toxin. This suggests the involvement of a permeability phenomenon. The studies by Kadis and Ajl (34) concerning the effect of toxin on mitochondrial swelling and the relationship between its respiration and swelling effects revealed that toxin induced rat heart and rat and rabbit liver mitochondria to swell but had no such effect on rabbit heart mitochondria (Table 2). Likewise, brain mitochondria exhibited very little spontaneous swelling and this was not affected by the addition of toxin (35). Thus, in every case where the toxin inhibits mitochondrial respiration, it induces swelling, and when it is incapable of inhibiting respiration, it is, likewise, unable to promote swelling.

Additional experiments (34) showed that, when the toxin is heat-inactivated or neutralized with antitoxin, it no longer induced swelling. This indicates that only toxin, active in vivo, can exert the in vitro swelling effect.

The mechanism of toxin-induced mitochondrial

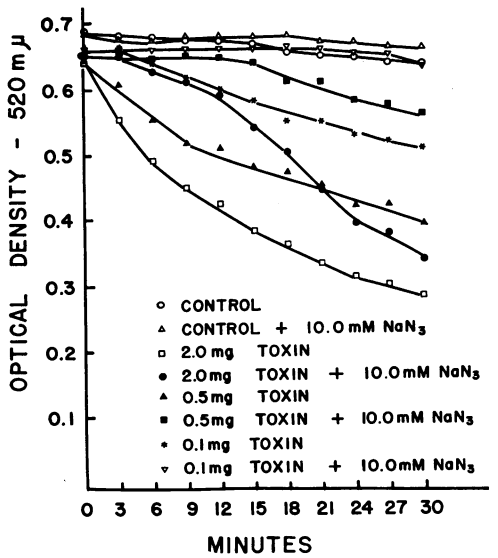

FIG. 2. *Effect of NaN_3 on toxin-induced swelling of rat heart mitochondria. From Kadis and Ajl (34).*

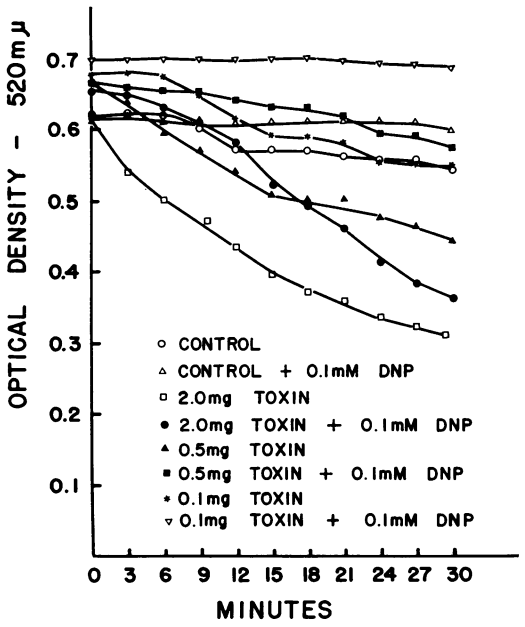

FIG. 3. *Effect of 2,4-dinitrophenol on toxin-induced swelling of rat heart mitochondria. From Kadis and Ajl (34).*

swelling was investigated by determining the effect of certain inhibitors of electron transport or high energy intermediate-supported swelling, such as cyanide (45), azide (30), and 2,4-dinitrophenol (70), on toxin-induced swelling. Each of these compounds prevented the swelling of rat heart mitochondria promoted by the toxin (Fig. 1 to 3). When rat heart mitochondria, for example, are incubated in an appropriate medium, a small but consistent degree of spontaneous swelling is observed. This spontaneous swelling is characterized by an initial, short lag period. The swelling curves in some of the toxin-induced swelling experiments together with the inhibitor studies showed that the above-mentioned lag periods were eliminated by the toxin and restored by cyanide, azide, and 2,4-dinitrophenol. This elimination of the lag periods by a swelling agent and their restoration by electron transport inhibitors is characteristic of electron transport-dependent swelling. The swelling caused by low concentrations of toxin was prevented completely by each of the inhibitors investigated. The partial prevention of swelling exhibited in the presence of high concentrations of toxin could be due to the damaging effect of large concentrations of toxin on the mitochondrial membrane, resulting in the elimination of electron transport.

Lehninger (43) observed that, whereas a large variety of chemical agents promoted swelling, only one agent, adenosine triphosphate (ATP), together with Mg^{++}, was responsible for mitochondrial contraction. In agreement with these findings, toxin-induced swelling was reversed by ATP plus Mg^{++}. Although ATP alone produced some initial reversal, this reversal effect rapidly decreased with time.

Effect on the electron transport system. At the time when it became apparent that a permeability phenomenon was involved in the action of this toxin, studies on the inhibitory effect of this toxin on mitochondrial respiration were reinitiated with the view towards pinpointing as precisely as possible the site of the inhibition. Since the toxin inhibited mitochondrial respiration but did not interfere with oxidative phosphorylation, it appeared logical that the toxin might exert its effect on the electron transport system. Kadis et al. (36) reported that, although the toxin inhibited the oxidation of α-ketoglutarate, succinate, malate, and β-hydroxybutyrate in the presence of ADP as phosphate acceptor, the percentage inhibition was, in no case, altered upon the addition of 2,4-dinitrophenol. In this respect the toxin did not behave, for example, like oligomycin, a classical inhibitor of oxidative phosphorylation. Lardy et al. (42) described the properties of oligomycin and other inhibitors of phosphorylating oxidation and reported that the inhibition of oligomycin was relieved by 2,4-dinitrophenol.

The cytochromes are among the chief components of the electron transport system, and each must remain in a reduced state for electrons to be transferred from reduced nicotinamide adenine dinucleotide ($NADH_2$) or succinate to oxygen, the terminal electron acceptor. A general indication can be obtained as to the site of action of the compound under investigation if, upon the examination of the absorption spectrum of a mitochondrial suspension to which has been added a compound whose mode of action is to be determined, an alteration can be found in one or more of the absorption peaks corresponding to specific cytochrome components.

Absolute and difference spectra of the cytochrome components of rat heart and liver mitochondria incubated in the absence and in the presence of toxin revealed that the addition of toxin caused the oxidation of cytochromes *a*, *b*, and *c*. This suggested that the toxin exerts its inhibitory effect on mitochondrial respiration by acting on the electron transport system in the region between $NADH_2$ or succinate and cytochrome *b*.

Confirmatory evidence on this point was obtained. The toxin had no effect on the oxidation of ascorbate by rat heart or liver mitochondria in the presence of tetramethylphenylenediamine

(TMPD) or cytochrome *c* as electron carriers (36). Since TMPD serves as a mobile electron carrier between ascorbate and members of the respiratory chain and acts between cytochrome *c* and oxygen (20), it appears that the toxin has no effect on the area of the electron transport system between cytochrome *c* and oxygen.

The next step in locating the precise site of action of the toxin on the electron transport system involved investigations on the effect of the toxin on the activity of enzymes known to occur between $NADH_2$ or succinate and cytochrome *b*. One of these enzymes is $NADH_2$ dehydrogenase. The specific activity of this enzyme, as assayed by the reduction of ferricyanide (54), in rat heart and liver mitochondria, as well as in electron transport particles prepared from rat heart, was not altered by the addition of toxin. This finding suggested that the toxin might not act on individual enzymes but on complexes of two or more respiratory carriers, representing limited segments of the electron transfer chain.

Four such complexes were isolated from beef heart mitochondria, and each one was obtained in highly purified form. Complex I corresponds to the $NADH_2$-coenzyme Q reductase of Hatefi et al. (26), which catalyzes the reduction of CoQ by $NADH_2$. Complex II refers to the succinic-coenzyme Q reductase of Ziegler and Doeg (80), which catalyzes the reduction of CoQ_2 and, to a considerably lesser extent, CoQ_{10} by succinate. Complex III is the reduced coenzyme Q-cytochrome *c* reductase of Hatefi et al. (27) that catalyzes the reduction of cytochrome *c* by reduced CoQ. Complex IV is the cytochrome oxidase system (19, 23) that catalyzes the oxidation of reduced cytochrome *c* by molecular oxygen. It should be noted that Hatefi et al. (25) discovered that complexes I to IV can be used as building blocks to reconstitute all or part of the electron transfer chain.

When purified $NADH_2$-cytochrome *c* reductase, from which $NADH_2$-coenzyme Q reductase and reduced coenzyme Q-cytochrome *c* reductase are derived, was incubated with toxin, its activity was inhibited to a significant extent (*unpublished data*). $NADH_2$-cytochrome *c* reductase activity of electron transport particles obtained from heavy beef heart mitochondria (ETP_H) was, likewise, inhibited by the toxin.

Similar results were obtained with $NADH_2$-coenzyme Q reductase. Since $NADH_2$ dehydrogenase is a major component of complex I, $NADH_2$-coenzyme Q reductase is capable of catalyzing the rapid reduction of ferricyanide by an amytal-insensitive reaction, a characteristic property of mitochondrial $NADH_2$ dehydrogenase. $NADH_2$-ferricyanide reductase activity of the purified complex I, as well as that from ETP_H, was not inhibited by the murine toxin of *P. pestis*. This indicates that the toxin has no effect on the activity of $NADH_2$ dehydrogenase and confirms the results of previous investigations (36) with intact mitochondrial suspensions. Additional evidence on this point stems from the fact that difference spectra of toxin-treated complex I revealed that the flavoprotein of this enzyme complex was as readily reduced in the presence of toxin as in its absence. Moreover, electron paramagnetic resonance spectroscopic studies on toxin-treated

TABLE 3. *Inhibition by toxin of Ca^{++} and P_i uptake by rat heart mitochondria and reversal by EDTA*[a]

Components present	Ca^{++} taken up[b]	P_i taken up[b]	Per cent inhibition	
			Ca^{++} taken up	P_i taken up
Complete system[c]	896	562	—	—
Plus 2.0 mg of toxin	473	292	47.2	48.0
Complete system (without substrate) plus 15 mM ATP	578	338	—	—
Plus 2.0 mg of toxin	261	172	54.8	49.1
Complete system including ascorbate and TMPD[d]	1028	602	—	—
Plus 2.0 mg of toxin	410	265	61.5	55.9
Complete system plus EDTA	1065	668	—	—
Plus 2.0 mg of toxin	879	550	17.5	17.6
Complete system including ascorbate and TMPD plus EDTA	1365	803	—	—
Plus 2.0 mg of toxin	1130	697	17.2	13.2

[a] This table was compiled from results given in reference 37.
[b] Expressed as millimicromoles per milligram of protein.
[c] Succinate was present as substrate.
[d] Reaction mixture included ascorbate plus TMPD instead of succinate.

complex I showed that the toxin in no way altered the nonheme iron content of the purified $NADH_2$-coenzyme Q reductase. Although the precise manner in which the toxin inhibits $NADH_2$-coenzyme Q reductase activity has not as yet been elucidated, the investigations on the effect of the toxin on the electron transport complexes suggest that the toxin exerts its inhibitory effect on the electron transport system by interfering with the enzymatic activity of $NADH_2$-coenzyme Q reductase, thus preventing coenzyme Q from being reduced.

Effect on mitochondrial ion accumulation. It has been shown that isolated mitochondria bind and accumulate K^+ (20), Mg^{++} (10, 12, 13), and Ca^{++} (11, 46, 64, 74, 75). Data suggested (13, 14, 20, 74) that alterations in the integrity of mitochondria may influence their ability to retain accumulated ions. Since plague murine toxin induces swelling, studies were initiated concerning the effect of the toxin on the accumulation of ions by mitochondria and the relationship between this effect and the ability of the toxin to induce mitochondrial swelling (37). Such knowledge should help in obtaining a better understanding of how the toxin influences myocardial physiology and eventually results in the death of a toxin-susceptible animal, because the absence or overabundance of ions results in abnormal states and reactions of the heart. It was found that the toxin inhibited the accumulation of Ca^{++} and inorganic phosphate (P_i) by rat heart mitochondria in the presence of succinate (Table 3) as well as α-ketoglutarate, malate, or β-hydroxybutyrate as substrate. Since it is known that the toxin can inhibit the oxidation of each of these compounds (62), it seemed conceivable that the toxin was preventing mitochondrial ion accumulation by interfering with the respiratory chain. That this was not the case was suggested by the finding that the toxin inhibits the ATP-supported accumulation of Ca^{++} and P_i by rat heart mitochondria in the absence of a respiratory substrate. Additional evidence on this point stemmed from the fact that, although toxin had no effect on the respiration of rat heart mitochondria in the presence of ascorbate and TMPD (36, 37), it inhibited the uptake of Ca^{++} and P_i supported by this segment of the electron transfer chain.

The relationship between the toxin's ability to induce swelling and to inhibit mitochondrial ion uptake was examined by incubating toxin-treated mitochondria with ethylenediaminetetraacetic acid (EDTA) at a concentration of 0.1 mM. This concentration of EDTA prevents swelling (70) without inhibiting ion uptake (75). The addition of EDTA largely prevented the inhibitory effect of the toxin on the accumulation of Ca^{++} and P_i by rat heart mitochondria in the presence of a respiratory substrate as well as in the presence of ascorbate and TMPD (see Table 3). These data suggested that EDTA, by preventing toxin-induced swelling, allowed mitochondria to retain ions that were accumulated in the mitochondrial lumen. However, it was also noted that EDTA-treated controls accumulated somewhat greater amounts of ions than untreated controls, indicating that EDTA could exert a general stabilizing effect on mitochondrial membranes.

DISTRIBUTION AND SYNTHESIS OF PLAGUE MURINE TOXIN

Location of the Toxin in the P. pestis Cell

Part of the general problem of toxin synthesis was the determination (55) of the location of toxin in *P. pestis* cells. At least 10% of total toxic activity was associated with the membrane fractions of spheroplasts prepared from whole cells; the remainder resided in the cytoplasmic fractions (Table 4).

Ribosomes obtained by high-speed centrifugation of the cytoplasmic fraction contained less than 1% of total toxin and total protein, suggesting that the cytoplasmic toxin of *P. pestis* exists as a nonconjugated, nonparticulate protein.

Membranes were disrupted by various means to examine the relationship of the toxin to the membrane of the *P. pestis* cell. A membrane suspension subjected to sonic vibration increased significantly the specific toxic activity of the protein compared with the original suspension (Table 4). Addition of magnesium ions to these suspensions protected the isolated membranes against

TABLE 4. *Distribution and release from membranes of toxin and total protein in Pasteurella pestis spheroplast fractions**

Spheroplast fraction	LD_{50} dose (μg of protein)	Total LD_{50} doses (toxic units)	Protein (per cent of total)
Cytoplasm	50	1,260	72
Membrane	162	152	28
Sonically treated membranes	79	80	45
Control	83	22	12
Sonically treated membranes plus $MgCl_2$	83	20	15
Control	83	19	9
Trypsin-treated membranes	109	40	60
Control	39	12	49

* This table was compiled from results given in reference 55.

disruption by sonic treatment. The LD_{50} of released protein decreased approximately threefold below the initial membrane-bound protein. These data suggest that the potential toxic activity of bound toxin cannot be adequately expressed until the toxin is solubilized.

When isolated membranes were incubated with trypsin, most of the protein released by trypsin action was nontoxic (Table 4). Since trypsin did not destroy the toxin, these findings suggested that the toxin may be bound in some manner which makes it inaccessible to trypsin action.

Resolution and Isolation of Two Toxic Components

Gel electrophoresis has been used in studying protein components in crude cell fractions for metabolic studies (28, 78) and in determining the purity of isolated proteins (50). While determining feasibility of this method for locating the toxin in crude cell fractions of *P. pestis*, toxin activity was observed to be associated with more than one protein component of the partially purified material. As demonstrated by the LD_{50}, increase in purity of a toxin sample was found to be related directly to a reduction in the total number of protein bands detected by gel electrophoresis (57). The purest samples obtained exhibited two bands, and each was found to be toxic when eluted and injected into mice.

Each protein had an LD_{50} for 16- to 18-g Swiss albino mice of 0.7 to 1.5 μg of protein and produced a single characteristic precipitation band when subjected to the gel diffusion precipitation technique. The slower migrating toxin, designated as toxin A, yielded a concave-shaped precipitin band, suggesting an antigen of greater molecular weight than the antibody (33). The more electrophoretically mobile toxin B, found to be identical with the earlier isolated toxin with a molecular weight of 74,000 (3),* revealed a straight or slightly convex precipitin band, suggesting a smaller molecular weight band than the antibody.

Protein patterns from extracts of membrane and cytoplasmic fractions obtained from lysed spheroplasts showed toxin A to be associated predominantly with the membrane and toxin B to be associated with cytoplasmic fraction. Toxin A is located in a different part of the *P. pestis* cell than toxin B, and toxin A appeared to be a larger molecular weight protein. This suggested two toxic proteins of different molecular species. This hypothesis was supported by data indicating differences in sensitivities of individual toxins to protein reagents such as deoxycholate, digitonin, urea, and various sulfhydryl reagents (57, 58).

On the other hand, it is speculated that both toxins are possibly located in, and are part of, the same particulate structure, namely, membranes in the native bacterial cell. It is proposed that during isolation, toxin B, located on the surface, splits off into the cytoplasmic fraction, whereas toxin A remains attached more strongly. It is also possible that toxin B is a part of the toxin A protein in the membrane; during stress of isolation, toxin A disaggregates to form an essentially "new protein," toxin B. This phenomenon was observed with bovine growth hormone (47) and glutamic dehydrogenase (72). The relative similarity in specific toxic activity of the two proteins suggests a structural relationship.

Recent evidence (*unpublished data*) points to some basic similarities in the amino acid content and ultraviolet spectra of the two toxins. Also there is a strong suggestion that toxin A is twice the molecular weight of toxin B, 240,000 and 120,000, respectively. This would suggest that the sensitivities of the two toxins to protein reagents is reflecting primarily differences in tertiary structure, and that toxin A is a dimer of toxin B.

Taking these data together, if the separation of the two toxins by cell location is not an artifact of isolation, then one could speculate that toxin B may be a precursor "polypeptide" of toxin A which is cemented into the membrane enzymatically after its dimerization. On the other hand, the possibility that toxin A is split in vivo to give two monomers of toxin B appears to be eliminated by results from some of the tryptophan analogue experiments subsequently discussed.

Selective Inhibition of Murine Toxin Synthesis by Tryptophan Analogues

In addition to studies on the location of murine toxin in *P. pestis*, experiments were also designed to determine mechanisms by which toxin synthesis is regulated. Toxin synthesis is selectively inhibited during growth of *P. pestis* at 37 C (55), and a number of metabolic inhibitors were examined in an attempt to separate toxin from total protein synthesis. The utilization of tryptophan analogues proved most effective for this purpose (56). A number of investigators reported the selective action of tryptophan analogues on protein synthesis (49, 63, 71). With these findings it became increasingly clear that tryptophan analogues may regulate protein biosynthesis in a specific and selective manner in addition to their role in end products inhibition.

* Recent re-examination of the molecular weight of toxin B by the Sephadex method has indicated a larger molecule of approximately 12,000 molecular weight.

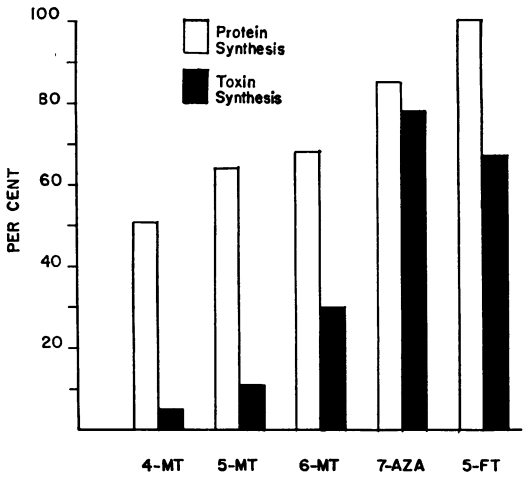

FIG. 4. *Selective action of tryptophan analogues on toxin formation. Results from reference 56.*

When various tryptophan analogues were incubated with *P. pestis* cells, it was found that, in all cases, toxin synthesis was much more sensitive to the action of the analogues than was general protein formation. The methyl analogues (4-, 5-, and 6-methyltryptophan) were more effective than were 7-aza- and 5-fluorotryptophan in inhibiting toxin and total protein synthesis (Fig. 4).

Antagonism of analogue bacteriostatic action and the selective inhibition of toxin. The addition of L-tryptophan, at a concentration as low as 1.0 μg/ml, to rapidly growing *P. pestis* cells was effective in reversing inhibition of growth and toxin synthesis by 4-methyltryptophan. Moyed and Friedman (59), using *Escherichia coli*, and Ames (6), using *Salmonella typhimurium*, reported permease competition between tryptophan and its analogues.

Three intermediates in the tryptophan biosynthetic pathway, i.e., indole, anthranilic acid, and shikimic acid, were used as antagonists of tryptophan analogue action (Table 5) to avoid possible permease competition in *P. pestis*, and to obtain unequivocal evidence for the hypothesis that the tryptophan synthetic pathway is the site of analogue inhibition. With indole the total protein and toxin inhibitory effects exhibited by 4- and 5-methyltryptophan and 5-fluorotryptophan were partially reversed by concentrations of indole lower than the analogue level. Anthranilic acid, however, produced a synergistic action in combination with 4-methyltryptophan; i.e., growth, total protein, and toxin were inhibited to a greater degree in *P. pestis* cells incubated with 4-methyltryptophan and anthranilic acid than in cells treated only with 4-methyltryptophan. Shikimic acid completely reversed the selective effect of 4-methyltryptophan on toxin synthesis but only partially prevented growth inhibition. The inability of anthranilic acid to reverse the inhibitory effect of 4-methyltryptophan is puzzling, because, from the data reported by Trudinger and Cohen (73) on the synthesis of anthranilate in mutants of *E. coli*, one would expect anthranilate to be a better antagonist than shikimate.

When tryptophan determinations were made on protein from analogue-treated and untreated *P. pestis* cells, it was found that the tryptophan content of the protein varied with the effectiveness of the inhibitors. Protein from cells treated with 4-methyltryptophan showed an 18% or more decrease in tryptophan. The tryptophan content of cells incubated with 4-methyltryptophan and indole was significantly higher than that of cells treated with 4-methyltryptophan alone. This paralleled the reversal of growth and toxin inhibition by indole.

Toxin protein content in extracts from analogue-treated cells. The toxin content of cell extracts from analogue-treated cells, cells treated with analogue plus indole, and control cells were compared to demonstrate the quantitative decrease in toxin synthesis in analogue-treated cells. Analogue addition in experiments lasting 4 to 5 hr resulted in a relative reduction in toxin A. However, extracts from analogue-treated cells re-

TABLE 5. *Indole, anthranilic acid, and shikimic acid as antagonists of the action of tryptophan analogues on total protein and toxin synthesis**

Sample	Protein formed	LD_{80} (μg of protein)
	mg	
Control	11.5	40
4-Methyltryptophan	5.3	60–80
4-Methyltryptophan + indole	8.9	40
Control	5.9	30
5-Methyltryptophan	4.3	60
5-Methyltryptophan + indole	5.7	40
Control	17.1	50
5-Fluorotryptophan	15.1	70
5-Fluorotryptophan + indole	15.9	60
Control	11.3	<40
4-Methyltryptophan	6.7	50
Anthranilic acid	12.1	<40
4-Methyltryptophan + anthranilic acid	4.1	>60
Control	9.9	<40
4-Methyltryptophan	4.7	60
Shikimic acid	8.3	40
4-Methyltryptophan + shikimic acid	4.9	40

* This table was compiled from results given in reference 56.

versed by indole exhibited a high concentration of toxin A, coinciding with the reversal of analogue inhibition. These results indicated that reduced toxin activity initiated by metabolic changes and detected by mouse assay is a result of the reduced amount of toxin A formed in analogue-treated cells. The possibility that the toxins are related and that toxin B is a metabolic end product, accumulating in the cytoplasm after release from the membrane, was suggested by investigations of Montie and Ajl (55) and by Csanyi et al. (15) with mammalian systems. If the membrane is a precursor of the cytoplasmic toxin, at the end of a 4- to 5-hr experiment, band B would be composed of the initial cytoplasmic toxin and initial membrane toxin transferred to the cytoplasm. The membrane toxin, according to this theory, would be composed of de novo synthesized toxin. Thus, longer term treatments beginning with small numbers of cells should result in the depletion of toxin B.

Dilute suspensions of cells were exposed to 5-fluorotryptophan for 8- to 11-hr periods (approximately 10 generations). This analogue was used because it caused little or no growth inhibition over a number of generations. When the extracts were assayed for toxic activity, it was found that the extracts of the analogue-treated cells exhibited a three- to fourfold increase in the LD_{50} as compared with that of the control cells. Toxin A was completely absent from alkaline extracts of treated cells, whereas toxin B was formed at rates comparable with the control cells. These results appeared to negate the precursor hypothesis.

Inhibitory effect of analogues on spheroplast toxin content. It was suggested (57) that toxin A is associated with the membrane fraction of the cell and toxin B with the cytoplasmic fraction. To confirm these data, intact cells were converted to spheroplasts in the presence of tryptophan analogues. Low concentrations of methyl analogues were employed to allow for complete conversion of whole cells to spheroplasts. When membrane and cytoplasmic fractions obtained from lysed spheroplasts were assayed for toxic activity, it was found that the membrane toxin was preferentially inhibited by the tryptophan analogues, whereas the cytoplasmic or soluble toxin was inhibited to the same degree as total protein. Consequently, these results present evidence confirming the identity of membrane-associated toxin with the tryptophan analogue-sensitive toxin A.

SUMMARY

During the past decade and a half or more, studies have been carried out on the murine toxin of *P. pestis*. A series of chemical and electrophoretic purification procedures yielded a most highly purified, serologically homogenous toxin. The amino acid composition and immunological properties of the purified toxin have been elucidated. Recent investigations revealed that this material consists of two distinct protein components, each of which possesses the same toxic activity and very similar amino acid content. Differences between the two toxic proteins are seen in their tertiary structure, molecular weight, cell location, and mode of biosynthesis. However, toxin B appears to be *one*-half the molecular weight of toxin A which suggests a monomer-dimer relationship between the two proteins.

Initial investigations on the mechanism of action of plague murine toxin carried out with cell-free microbial extracts and crude tissue homogenates showed that α-keto acid oxidation is specifically inhibited, and that this inhibition is reversed with excess NAD but not with NADP. The most significant findings, however, involved the effect of the toxin on mammalian mitochondria. A correlation has been established between the ability of the toxin to inhibit heart mitochondrial respiration of certain animal species and the susceptibility of these species to the in vivo action of the toxin.

The inhibitory effect on mitochondrial respiration is exerted on a segment of the electron transport system between $NADH_2$ or succinate and cytochrome *b* and, more specifically, at the level of $NADH_2$-coenzyme Q reductase.

In addition, plague murine toxin induces mitochondria to swell and curtails the accumulation of calcium and inorganic phosphate ions by these structures. A direct relationship between these effects has been established. By virtue of its ability to alter the integrity of intact mitochondria, the toxin does not allow the ions that have been accumulated in the mitochondrial lumen to be retained.

Investigations on toxin biosynthesis revealed that this process is inhibited by tryptophan analogues which inhibit tryptophan biosynthesis, and that this inhibition is reversed by intermediates of the tryptophan biosynthetic pathway in microorganisms, suggesting that tryptophan is required for the biosynthesis of toxin.

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